Supplementary Material 2: Step-by-step Experimental Protocol

Whole Mount *In Situ* Hybridization of Mouse Early Embryo with DIG-labeled RNA Probe

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High-throughput screen for genes predominantly expressed in the ICM of mouse blastocysts by whole mount *in situ* hybridization

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MATERIALS

1. Aluminum Chamber system (see Supplementary Material 1)
2. Transwell, 6.5 mm diameter, 8.0 μm pore size, polycarbonate membrane, Corning (Cat. 3422)
3. Center-well organ culture dish (Falcon #3037)
4. PBS: Phosphate buffered saline
5. PBT: PBS, 0.1% Tween-20
6. 4% PFA/PBT: 4% Paraformaldehyde in PBT
7. PK/PBT: Proteinase K in PBT. PK concentration should be optimized for each batch. For example, we used [PK] = 3.0 μg/ml (Roche Cat #3115828; Lot #11009300, 20 mg/ml), which means 3.75 μl/25 ml PBT.
8. 4%PFA/0.2%GA/PBT: 4% Paraformaldehyde, 0.2% EM-grade Glutaraldehyde in PBT
9. Hybridization Buffer (HB): 4X SSC (pH 7.0), 50% deionized Formamide, 100 μg/ml Heparin, 250 μg/ml Yeast tRNA, 100 μg/ml Salmon Sperm DNA, 2X Denhardt's solution, 0.1% Tween-20.
10. Post-hybridization Wash Buffer (PHWB): 50% Formamide, 2X SSC, 0.1% Tween-20.

PREPARATION OF STOCK SOLUTION

4% Paraformaldehyde (PFA)/ PBT

DEPC water	-	24 ml
10x PBS	1x	3.6 ml
3% Tween 20	0.1%	1.2 ml
16% Paraformaldehyde	4%	9.0 ml
	36 ml	Use 1 ml for each transwell

PBT (Phosphate buffered saline with 0.1% Tween-20)

DEPC water	-	43.3 ml	130 ml	151.67 ml
10x PBS	1x	5.0 ml	15 ml	17.5 ml
3% Tween-20	0.1%	1.67 ml	5 ml	5.83 ml
		50.0 ml	150 ml	175.0 ml

Proteinase K (PK)/PBT 3.0 µg/ml (Roche Cat #3115828, Lot #11009300)

PBT	-	25 ml
Proteinase K stock (20 mg/ml)	3.0 µg/ml	3.75 µl
		25.4 ml

Hybridization buffer (HB) [Store at -20 °C]

DEPC water	-	3.3 ml	6.6 ml
20x SSC (pH7.0)	4x	4.0 ml	8.0 ml
Formamide (deionized)	50%	10 ml	20.0 ml
10 mg/ml Heparin	100 µg/ml	0.2 ml	0.4 ml
10 mg/ml tRNA	250 µg/ml	0.5 ml	1.0 ml
10 mg/ml salmon sperm DNA	100 µg/ml	0.2 ml	0.4 ml
50x Denhardt's solution	2x	0.8 ml	1.6 ml
3% Tween-20	0.1%	0.7 ml	1.4 ml
		20.0 ml	40.0 ml

DIG-labeled riboprobe / Hybridization buffer (1 µg/ml)

Hybridization buffer[1]	-	300 µl	
DIG-labeled riboprobe Stock	1 µg/ml	1.0 µl – 2.5 µl	
		301 µl - 302 µl	Use 700 µl for each transwell

Post-hybridization Washing Buffer (PHWB)

Formamide	50%	45.0 ml	50.0 ml	67.5 ml
20x SSC	2x	9.0 ml	10.0 ml	13.5 ml
3% Tween-20	0.1%	3.0 ml	3.3 ml	4.5 ml
MQ Water	-	33.0 ml	36.7 ml	49.5 ml
		90.0 ml	100.0 ml	135.0 ml

Blocking solution

10x blocking solution	1x	3 ml	
1x Maleic acid buffer	-	27 ml	
		30 ml	leave aliquot for Ab solution

AP conjugated anti-DIG Ab / Blocking buffer

Blocking solution	-	1 ml	2 ml	
AP conjugated anti-DIG Ab	x2000	0.5 µl	1 µl	
		1 ml	2 ml	Use 100 µl for each transwell

Detection buffer

MQ water	-	88.0 ml .
5M NaCl	100 mM	2.0 ml
1M TrisHCl (pH9.5)	100 mM	10.0 ml
		100.0 ml

NBT; BCIP / Detection buffer

Detection buffer	-	4.9 ml	9.8 ml	
NBT ; BCIP	1:50	0.1 ml	0.2 ml	
		5.0 ml	10.0 ml	Use 500 µl each transwell

PROCEDURES

A. Embryo Collection and Fixation

1. Harvest embryos in cold PBS in Center-well organ culture dish (Falcon #3037) in a standard manner.
2. Pipette 0.9 ml of 4% PFA/PBT in each insert of Transwell plate (Corning #3422: 24-well). Place the plate on ice.
3. Transfer embryos by a mouth-pipette into each insert. Keep the plate on ice or at 4 ℃ for 30 min – overnight for fixation.

B. Pretreatments

1. Transfer each insert to wells of the Aluminum Chamber System (see Supplementary Material 1). All the procedures from here will be done using this Chamber System. Solution will be added to each insert by pipetting and drained by the capillary action.
2. Rinse embryos once with PBT for 2 min at RT
3. Wash embryos twice with PBT for 5 min each at RT with gentle agitation on a shaker.
4. Treat embryos with PK/PBT for 15 min at RT with very gentle shaking. Note: We recommend that each batch of PK should be tested for optimal concentration (0.5 – 10.0 μg/ml) using Pou5f1 (Oct3/4).

5. Rinse with PBT once for 2 min at RT.

6. Postfix embryos in 4% PFA/0.2% GA/PBT for 20 min at RT with shaking. Note: After PK treatment, embryos become very fragile. It is necessary to fix the embryos with stronger fixation reagents than paraformaldehyde, such as glutaraldehyde.

7. Rinse with PBT once for 2 min at RT; Wash with PBT twice for 5 min each at RT with shaking.

C. Prehybridization, Hybridization, and Post-hybridization Wash

1. Prehybridize embryos in 500 μl hybridization buffer (HB)/well at 60 ℃ for 3 hrs–8 hrs. Note: Hybridization temperature has great influence on the result of WISH. For highthroughput application, we normally use 60 °C. However, if it is possible, we recommend that optimum temperature should be found by testing 60 °C, 65 °C, and 70 °C.

2. Add DIG-labeled probe (1.0 μl or 5.0 μl) to 200 μl HB and transfer them to wells. Hybridize embryos at 60 °C (or 65 °C, and 70 °C) for overnight.

3. Rinse with pre-warmed Post-hybridizaton Wash Buffter (PHWB) once for 2 min. Wash with PHWB 4 times for 25 min each with shaking. Both rinsing and washing are carried out in heat block at the same temperature of hybridization. Turn off heat block for the last wash to allow embryo to cool down gradually to RT. Note: During the transition from hybridization to post-hybridization wash, it is important to maintain the temperature.

D. Immunocytochemical Detection of DIG-labeled Probes

1. Rinse with Washing Buffer once for 2 min at RT. Wash once with Washing Buffer for 10 min at RT with shaking.

2. Rinse with 1X Blocking Solution once for 2 min at RT. Leave embryos in Blocking Solution for 30 min at RT with shaking. Note: 1X Blocking Solution needs to be made fresh each time.

3. Incubate embryos with Anti-DIG AP-conjugate antibody in 1X Blocking Solution for 1- 2 hrs at RT with shaking. Note: If embryos are in sealed tubes or in enough solution, this step can be done at 4 °C for overnight. A key is not to let embryos dry.

4. Rinse once with Washing Buffer for 2 min at RT. Wash with Washing buffer for 10 min at RT with shaking.

5. Wash with Detection Buffer twice for 10 min each at RT with shaking.

6. Transfer inserts from the Chamber System to a regular 24-well plates.

7. Add 500 µl/well NBT/BCIP in Detection Buffer to each insert/well and incubate embryos in dark for 30 min– 24 hrs at RT with shaking for the first 15 min. Note: Abundant RNA, such as Actb, should be visible after 30 minutes.

8. Transfer inserts back to the Chamber System.

9. Stop reaction by rinsing embryos with PBT three times for 5 min each at RT with shaking. Store embryos in PBS containing 1 mM EDTA at 4 °C. The staining pattern should be stable at least for several weeks.

E. Photographing

1. Transfer embryos from insert to a 24-well plate by pipetting with 200 µl PBS containing 1 mM EDTA and 20% glycerol.

2. Take a photograph with bright field under 20X objective lens. If it is desirable to photograph embryos on a glass slide or for long-term storage, embryos can be transferred from insert to slides as follows: After drainage of PBT from the inserts, melted (55 °C) glycerol gelatin (Sigma) is added to inserts and then allow glycerol gelatin to solidify at 4 °C. After removing the polycarbonate membrane bottom of the inserts using forceps, inserts are placed on glass slides, which are then placed on a heat block at 55 °C for a few minutes. After glycerol gelatin is melted, we remove the insert, place on cover glasses and allow glycerol gelatin to solidify again.

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